Analysis of selected *Crinum* species for galanthamine alkaloid: an anti-Alzheimer drug

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Galanthamine, an isoquinoline alkaloid and the current drug of choice for treatment of mild to moderate Alzheimer disease, is mainly obtained from Amaryllidaceae members. At present, the production of galanthamine from available natural plant sources is not sufficient to meet the demands of pharmaceutical industry. Additionally, overexploitation of the plant material leads to the depletion of available natural wild populations. Therefore, there is a need to explore additional natural sources for the extraction of this drug. Thus the aim of this study is to determine galanthamine content in five Indian *Crinum* species. The bulbs of each *Crinum* species were extracted with methanol. Extracts were analysed by high performance liquid chromatography. The chromatographic separation was performed using an isocratic system with a mobile phase of methanol : 5 mM $(NH_4)_2HPO_4$ (55 : 45 v/v) applied at a flow rate 0.8 ml/min using a UV detector at 288 nm. Among all the *Crinum* species studied, the highest galanthamine content was found in the bulbs of *C. malabaricum* Lekhak & S.R. Yadav (0.308 ± 0.004%), followed by *C. viviparum* (Lam.) R. Ansari & V.J. Nair (locality Ratnagiri; 0.262 ± 0.042%). However, *C. brachynema* Herb. and *C. pratense* Herb. (locality Borbet) showed the lowest and equal galanthamine content (0.029 ± 0.000%) in their bulbs. Galanthamine was not detected in the bulbs of *C. latifolium* L. This study identifies novel plant sources of galanthamine, which may be helpful for pharmaceutical production of galanthamine. The present study provides a quantitative comparison of galanthamine among Indian *Crinum* species.

Keywords: Alzheimer disease, chromatography liquid, *Crinum* species, galanthamine.

GALANTHAMINE (sometimes referred to as galantamine) is an isoquinoline Amaryllidaceae alkaloid and FDA approved drug (Razadyne® in USA, Reminyl® in Europe) for symptomatic treatment of patients with mild to moderate Alzheimer disease[1,2]. An extensive clinical trial in patients with Alzheimer disease has shown that galanthamine has broad, sustained benefits for at least 52 weeks in cognitive and functional abilities, global response and caregiver burden[3]. Galanthamine is a selective, reversible, competitive inhibitor of acetylcholine esterase and is isolated from various Amaryllidaceae plants belonging to the genera *Galanthus*, *Leucojum*, *Lycoris*, *Narcissus* and *Ungernia*[4–6]. Recent developments in the use of natural products as therapeutics for Alzheimer disease were reviewed by Williams *et al.*[7] Alzheimer disease is the most common form of dementia, and estimates suggest that 15 million people worldwide are affected by this disease[8]. However, scarce supplies from threatened botanical sources with limited regeneration, unsuccessful cultivation and expensive isolation processes are major constraints to meet the increasing demand of the drug. Hence several methods of chemical synthesis were developed to produce this drug, but again these are expensive, multistep processes limited by low yield. According to the World Health Organization, 80% of the world population in the developing countries depends on traditional medicines obtained from natural plants for primary healthcare needs[9]. Natural plant resources are the primary source of structurally diverse natural compounds exhibiting different bioactive properties leading to the development of innovative and effective drug molecules[10]. Thus, wild plants remain as untapped sources for identification and isolation of galanthamine and other important biomolecules.

The genus *Crinum* comprises approximately 104 species distributed throughout the tropics and warm temperate regions of the world in Asia, Australia, Africa and America. In India it is represented by 14 species, 1 variety and 1 form of which 5 are endemic to the country[11]. The *Crinum* species have significant and desirable ornamental, commercial, economic and medicinal importance. In addition, *Crinum* plants have been found to contain many alkaloids with pharmacological activity, including galanthamine[12–14]. To our knowledge, no studies on phytochemical analysis with reference to galanthamine and other alkaloids content have been carried in *Crinum* species confined to the Indian region.

Against this background, a few species of *Crinum* belonging to the Amaryllidaceae family were analysed quantitatively by HPLC for the determination of galanthamine.

The bulbs of wild plants of *Crinum* species were collected from various localities in India (Table 1). The voucher specimens are deposited in the herbarium of the Botany Department, Shivaji University, Kolhapur, India. The bulbs were planted in the pots and live collections are maintained in the Botanical Garden of Shivaji University. The bulbs of individual species were cut into small pieces, dried at 60°C for 24 h to constant weight and then powdered using a mechanical grinder. The powdered plant material was stored in separate plastic containers at room temperature until further analysis.

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Table 1. *Crinum* accessions used in this study and galanthamine content in bulbs (expressed as % DW ± SD)

Crinum species	Locality	District	Latitude (N)	Longitude (E)	Altitude (feet)	Galanthamine (%; DW)
C. brachynema Herb.	Kates point	Satara	17°55′841″	73°42′236″	4348	0.029 ± 0.000
C. latifolium L.	Vandre	Pune	18°31′019″	73°26′664″	2027	ND
C. malabaricum Lekhak & S.R. Yadav	Periya	Kasaragod	12°24′526″	75°06′571″	333	0.308 ± 0.004
C. pratense Herb.	Borbet	Kolhapur	16°31′070″	73°53′586″	3140	0.029 ± 0.000
C. pratense Herb.	Barki	Kolhapur	16°44′673″	73°50′824″	3212	0.132 ± 0.002
C. viviparum (Lam.) R. Ansari & V.J. Nair	On the way to castle rock	Belgaum	15°26′314″	74°29′234″	2015	0.055 ± 0.000
C. viviparum (Lam.) R. Ansari & V.J. Nair	MIDC Ratnagiri	Ratnagiri	16°52′793″	73°19′593″	235	0.262 ± 0.042
C. viviparum (Lam.) R. Ansari & V.J. Nair	Belgaum	Belgaum	15°40′392″	74°07′021″	2615	0.046 ± 0.000

ND, Not detected.

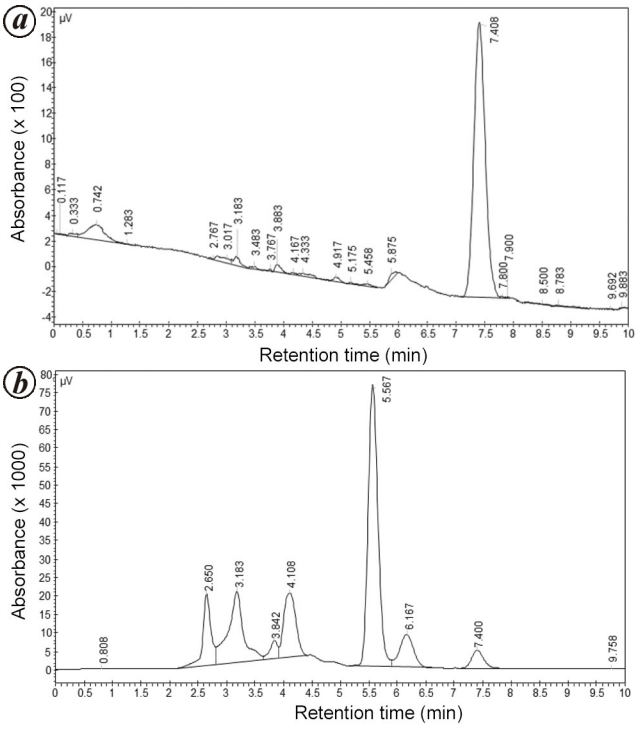

Figure 1. HPLC chromatograms of galanthamine authentic sample (***a***) and *Crinum* species bulb extract (***b***) detected using Innertsil C18 column and UV absorbance monitored at 288 nm utilizing methanol: 5 mM, $(NH_4)_2HPO_4$ (55 : 45) as mobile phase at a flow rate of 0.8 ml min^{-1}.

All the reagents and solvents used during the experiment were of analytical grade and highest purity – galanthamine hydrobromide and dimethyl sulphoxide (DMSO; Sigma, India), $(NH_4)_2HPO_4$ (S.D. Fine Chemicals Ltd, India) and HPLC-grade methanol (Merck, India).

The bulbs of wild plants of *Crinum* species were harvested and analysed separately for isoquinoline alkaloid galanthamine content. Dried and powdered material was mixed with 1 ml of methanol and sonicated at 33 kHz for 10 min at room temperature. Samples were centrifuged at 14,000 *g* for 10 min and supernatant was directly checked using HPLC for galanthamine content.

The extract was quantified by HPLC. The analysis was performed on Jasco Liquid Chromatograph (model 980, Japan) equipped with auto sampler injector (model no. Jasco AS-950, Japan) with a 25 µl loop and a variable wavelength detector (model no. UV-975, Japan). Data collection and integration were accomplished using BORWIN software. Separations were performed on Inertsil C18 (5 µm, 250 × 4.6 mm ID, Sigma, USA) column. Galanthamine was determined using methanol : 5 mM, $(NH_4)_2HPO_4$ (55 : 45 v/v) as the mobile phase. The flow rate was 0.8 ml/min and elution was monitored at 288 nm (Figure 1). Validation of quantitative method was performed by injecting five different concentrations of the same sample. The results of the five injections from the same samples at five concentrations (0.01–0.5 µg) showed similar retention time. The analytical operation was completed in 15 min.

Analyses and measurements of each sample were repeated three times and average values were used for further statistical evaluation. The experiment was conducted using five bulbs from each species. The results are presented as means ± SD. The data were analysed statistically by analysis of variance (ANOVA) and difference between means of the samples was analysed by the least significant difference (LSD) at a probability level of 0.05.

The galanthamine content of *Crinum* species is shown in Table 1. All the screened species were found to contain galanthamine alkaloid in the bulbs, except *C. latifolium* L. Among all *Crinum* species studied, the highest galanthamine content was found in the bulbs of *C. malabaricum* Lekhak & S.R. Yadav (0.308 ± 0.004%), followed by *C. viviparum* (Lam.) R. Ansari & V.J. Nair (locality Ratnagiri) (0.262 ± 0.042%). However, *C. brachynema*

Herb. (locality Kates point) and *C. pratense* Herb. (locality Borbet) showed the lowest and equal galathamine content (0.029 ± 0.000%) in their bulbs. Earlier reports in the literature have shown that *C. powelli* Hort., *C. lawrentii*, *C. defixum* Keraudren & Gawl. and *C. asiaticum* L. contain galanthamine alkaloid[13] and among the *Crinum* species studied, galanthamine yield was in the range 0.0–0.043% (ref. 15). The interspecific variations with respect to galanthamine alkaloid were observed in ornamental varieties of *Narcissus*[6]. The wide intraspecific variations in galanthamine content of *C. viviparum* (Lam.) R. Ansari & V.J. Nair collected from different geographical localities were observed in the present work (Table 1). No significant differences were detected between the bulbs of *C. brachynema* Herb. and *C. pratense* Herb. as far as galanthamine content is concerned.

The present results reveal that *C. viviparum* (Lam.) R. Ansari & V.J. Nair collected from Ratnagiri area and growing at lower altitude (235 ft) accumulated high amount of galanthamine compared to similar species collected from higher altitude (2015 and 2615 ft). The intraspecific variation was also observed in *C. pratense* Herb. This might be due to the differences in ecological factors, such as altitude, illumination, temperature, humidity and soil, which influence the type and contents of secondary metabolites in medicinal plants[16]. Similarly, the geographically isolated populations of *Leucojum aestivum* L. have led to differences in the alkaloid biosynthesis and consequently to the occurrence of different chemotypes[17].

This study reported *Crinum* species as a source of galanthamine in addition to other Amaryllidaceae members. Some of these species yield comparable amounts of galanthamine, and can be screened further for commercial extraction of galanthamine, although most of them are being used as traditional medicines for various purposes. This offers incentive for their conservation and improvement by conventional or biotechnological approaches, as they are threatened by increasing urbanization and transformation of pharmaceutically important *Crinum* species into valued commodities.

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